International Immunopharmacology 11 (2011) 199–203

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Oral administration of heat-killed *Lactobacillus pentosus* strain b240 augments protection against influenza virus infection in mice

Noritada Kobayashi [a], Takao Saito [b,*], Takayuki Uematsu [a], Kazumasa Kishi [b,1], Masamichi Toba [b], Noriyuki Kohda [b], Tatsuo Suzuki [a]

[a] Biomedical Laboratory, Division of Biomedical Research, Kitasato Institute Medical Center Hospital, Kitasato University, Saitama, Japan
[b] Otsu Nutraceuticals Research Institute, Nutraceuticals Division, Otsuka Pharmaceutical Co., Ltd., Shiga, Japan

ARTICLE INFO

Article history:
Received 13 October 2010
Accepted 6 November 2010
Available online 21 November 2010

Keywords:
Influenza virus
Mice
Lactobacillus pentosus strain b240
Immunoglobulin A

ABSTRACT

Host-defense mechanisms against influenza virus (IFV) infection involve both innate and acquired immunities. Among other components, secretory immunoglobulin A (SIgA) in the airway mucosa plays a particularly pivotal role in preventing IFV infection. Among 150 strains of lactic acid bacteria, *Lactobacillus pentosus* strain b240 (b240) has the highest IgA-inducing potency in mouse Peyer's patch cells. We previously reported a practical new finding that oral ingestion of nonviable heat-killed b240 elevates salivary IgA secretion in humans. The present study aimed to determine if nonviable b240 can prevent IFV infection in mice.

In a BALB/c mouse model infected with lethal levels of IFV A/PR8/34 (H1N1), oral administration of b240 for 3 weeks by gavage prior to IFV infection significantly prolonged the survival period. For IFV infection at nonlethal levels, the infectious titers of IFV in the lungs 7 days after infection were significantly reduced after similar b240 administration. Both anti-IFV IgA and immunoglobulin G titers in bronchoalveolar lavage fluid and plasma on day 7 were significantly higher in the b240-treated group than the control group. The augmentation of the anti-IFV immune response by b240 application was preliminarily confirmed by the elevated production of IFV-driven T-cell factors during mixed lymphocyte reactions with b240-primed splenocytes.

These results suggest that oral nonviable heat-killed b240 intake can facilitate protection against IFV infection.

1. Introduction

Massive outbreaks of influenza virus (IFV) infection can occur seasonally, and fatalities are high when IFV infection affects infants, young children, and the elderly [1]. Although various medical interventions such as administration of anti-IFV agents and vaccination have been undertaken worldwide, a comprehensive method to prevent outbreaks remains to be established [2]. Anti-IFV agents such as neuraminidase and M2 proton channel inhibitors are widely used to treat IFV infection [3]. However, these medications have limitations, including the need to be administered within 48 h of infection for efficacy [4], adverse reactions [5], and inability to cope with viruses resistant to these drugs [6].

Vaccination has been recommended and implemented as a means of preventing IFV infection [2]. At present, vaccines injected subcutaneously or intramuscularly induce immunoglobulin G (IgG) specific to the vaccinated strain. However, they may not be sufficiently efficacious against infections by viral strains other than the vaccination strain, especially against those with alien viral antigenicity [7]. Furthermore, although an elevated blood IgG level may reduce the symptoms after infection, it is not sufficient to prevent infection [8]. In contrast, mucosal vaccines administered via nasal or oral routes can induce secretory immunoglobulin A (SIgA), which has broader cross-activity than IgG, locally in the airway mucosal epithelium [9]. Mucosal vaccines can cross-react with viral strains that differ from the vaccination strain, and they can be used for prophylaxis [10]. Therefore, mucosal vaccines are being actively developed [11,12].

Lactobacillus pentosus strain b240 has the highest IgA-inducing potency among 150 strains of lactic acid bacteria in mouse Peyer's patch cells [13]. In clinical settings, the intake of b240 for 3 [14] or 12 weeks [15] results in a significant increase in SIgA secretion in the saliva, without any attendant side effects. This ability to complement the mucosal immune response by augmenting SIgA production prompted

Abbreviations: IFV, influenza virus; SIgA, secretory immunoglobulin A; IgG, immunoglobulin G; MDCK, Madino–Darby canine kidney; b240, *Lactobacillus pentosus* ONRIC b0240; PBS (+), phosphate-buffered saline with $MgCl_2$ and $CaCl_2$; $TCID_{50}$, tissue culture infectious dose 50; BALF, bronchoalveolar lavage fluid; PBST, 1% BSA–PBS containing 0.05% Tween-20; NK, natural killer; IFN, interferon; NW, nasal wash fluid.

* Corresponding author. Otsu Nutraceuticals Research Institute, Nutraceuticals Division, Otsuka Pharmaceutical Co., Ltd., 13-31-3, Saigawa, Otsu, Shiga, 520-0002, Japan. Tel.: +81 77 521 8835; fax: +81 77 525 1135.
E-mail address: taka_saito@research.otsuka.co.jp (T. Saito).
[1] Present address: Therapeutic Application Department Development, Pharmaceutical Business Division, Otsuka Pharmaceutical Co., Ltd.

1567-5769/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2010.11.019

us to explore the capability of b240 to provide *in vivo* protective effects against various infectious diseases. The effectiveness of the oral administration of heat-killed b240 has been previously confirmed in mouse models of *Salmonella* infection [16].

This study was conducted to evaluate if the oral administration of heat-killed nonviable b240 augments the host-defense mechanisms against IFV infection in mice.

2. Materials and methods

2.1. Bacteria

L. pentosus strain b240 (ONRIC b0240: b240) was isolated by Okada et al. from fermented tealeaves [17]. Strain b240 was initially identified as *L. plantarum*. Recently, Bringel et al. proposed reclassification of the *L. plantarum* group on the basis of the *recA* gene sequence [18]. Following their proposal, we confirmed b240 as *L. pentosus*.

Strain b240 was grown in MRS broth (Beckton Dickinson, Franklin Lakes, NJ) at 33 °C for 24 h. The culture was washed twice with saline and suspended in de-ionized water, followed by autoclaving at 121 °C for 15 min. The heat-killed bacterial suspension was freeze-dried for storage. It was resuspended in saline before use.

2.2. Cells

Madino–Darby canine kidney cells (MDCK, RCB0995, RIKEN, Ibaragi, Japan), a canine kidney epithelial cell line, was grown in Eagle's minimum essential medium (Gibco; Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). CTLL-2 (RCB0637, RIKEN, Ibaragi, Japan) was grown in RPMI 1640 medium (Gibco; Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum and 50 U/mL recombinant mouse IL-2.

2.3. Virus

MDCK cells were infected with IFV A/PR8/34 H1N1 at a multiplicity of infection of 0.01 and cultured at 37 °C for 72 h in a 5% CO_2 atmosphere (passage 1). Cells continuously cultured for 5 passages were stored at −80 °C.

To prepare the IFV, frozen IFV-infected MDCK cells were subjected to 3 cycles of freezing and thawing, followed by centrifugation for 15 min (1500×*g*, 4 °C). The supernatant was combined with polyethylene glycol 6000 (Nacalai Tesque, Inc., Kyoto, Japan) at a final concentration of 8% w/v. The mixture was gently agitated for 6 h at 4 °C, followed by centrifugation for 30 min (15,000×*g*, 4 °C). The sediment was moistened with NTE buffer (0.1 M NaCl, 0.01 M Tris–hydrochloride, 0.001 M EDTA, pH 7.4) and combined with phosphate-buffered saline with $MgCl_2$ and $CaCl_2$ (PBS (+)). The mixture was then agitated upside-down on a rotator (4 °C, 16 h), followed by centrifugation for 30 min (15,000×*g*, 4 °C). The supernatant was layered onto a sucrose density gradient (10–40% w/v) and centrifuged for 90 min (82,000×*g*, 4 °C). The IFV fraction was harvested and combined with PBS (+), followed by layering on a sucrose density gradient (30–60% w/v) and centrifugation for 150 min (82,000×*g*, 4 °C). The IFV fraction was harvested and combined with the cell culture medium, followed by centrifugation for 90 min (82,000×*g*, 4 °C). The sediment was combined with PBS (+) to yield an IFV suspension. The viral fluid was stored at −80 °C prior to use. Part of the fluid was diluted 10-fold to confirm cell degeneration, thereby determining the tissue culture infectious dose 50 ($TCID_{50}$) [19,20].

2.4. Mice

Five-week-old female BALB/c/Cr Slc (SPF) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and acclimatized for 1 week. Mice were maintained in polycarbonate cages (CLEA Japan, Inc., Tokyo, Japan) at 23 ± 2 °C, a relative humidity of 55 ± 15%, and under a 12-hour light cycle (illuminated from 8:00 to 20:00 h). The mice were allowed free access to CRF-1 pellets (Oriental Yeast Co., Ltd., Tokyo, Japan) and autoclave-sterilized water. The animal experiments were performed according to the animal care guidelines of the Kitasato Institute Medical Center Hospital.

2.5. Lethal infection model

Six-week-old BALB/c mice were divided into 4 groups of 10 mice each on day 1 of the study. Strain b240 diluted in saline was administered daily by gavage at doses of 0.4, 2, or 10 mg/mouse during the experimental period. The dosages were determined based on previous studies on the enhancement of SIgA secretions in the intestines. The control group received saline by gavage. On day 22, the mice were anesthetized by intramuscular injections of 25 mg/kg ketamine hydrochloride (50 mg/mL, Sankyo Co., Ltd., Tokyo, Japan) and 0.625 mg/kg droperidol (2.5 mg/mL, Sankyo Co., Ltd.) to the hind legs with. Then, 50 μL of $10^{7.5}$ $TCID_{50}$/mL of IFV diluted in PBS was inoculated into the right nasal cavity of each mouse. Survival of the mice was monitored daily for 2 weeks after IFV infection.

2.6. Nonlethal infection model

On day 1 of the study, 6-week-old BALB/c mice were divided into 5 groups: 4 IFV-inoculated groups of 25 mice each and 1 IFV inoculation-free group of 3 mice. In the 4 IFV-inoculated groups, each mouse was administered 0.2 mL of 0.4, 2, or 10 mg of b240 or saline by gavage every day from day 1 to the day of autopsy. On day 22, $10^{7.25}$ $TCID_{50}$/mL IFV was administered nasally in the same manner as the lethal infection model. On day 25 (3 days after infection), 27 (5 days after infection), and 29 (7 days after infection), blood was sampled from the inferior vena cava of 5 mice in each IFV-inoculated group, and the spleen of 1 mouse from the IFV inoculation-free group was harvested.

On day 29 (7 days after infection), bronchoalveolar lavage fluid (BALF) was collected from 10 mice in each IFV-inoculated group. Then, blood was sampled from the inferior vena cava, and plasma was obtained. Furthermore, the lungs of each mouse were harvested. The BALF and plasma were stored at −20 °C. The lungs were frozen in liquid nitrogen and stored at −80 °C until analysis.

2.7. Anti-IFV antibody titer

Each well of ELISA plates (96-well flat-bottom plates, Nalge Nunc International K.K., Tokyo, Japan) was coated with 50 μL inactivated IFV suspension (infectious titer before inactivation equivalent to $10^{7.0}$ $TCID_{50}$/mL) at 4 °C for 16 h. After blocking with 50 μL/well of 1% BSA–PBS containing 0.05% Tween-20 (PBST) (Sigma-Aldrich Corp., St. Louis, MO), the samples (50 μL/well) were applied to the plate and incubated at 37 °C for 1 h. The plates were washed 3 times with 300 μL/well of PBST, and 50 μL/well of either 2000-fold diluted peroxidase-conjugated goat affinity purified antibody to mouse IgG (MP Biomedicals LLC., Solon, OH) or peroxidase-conjugated goat IgG fraction to mouse IgA (alpha chain, MP Biomedicals LLC) was added. After incubation for 1 h at room temperature, the plates were washed as described above, combined with 50 μL/well of FAST OPD (Sigma-Aldrich Corp., P9187), and incubated at room temperature for 15 min with light shielding. The mixture was then combined with 50 μL/well 4 N H_2SO_4 to stop the reaction, and the OD_{490} was measured with a microplate reader (Benchmark, Bio-Rad Japan, Tokyo, Japan).

2.8. IFV infectious titer in lung

A frozen lung was homogenized with a Teflon homogenizer to prepare a lung suspension. The suspension was serially diluted 10-fold